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Antimicrobial and Modulatory Activity of Ethanol Extract of the Leaves from *Lygodium venustum* SW.

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Abstract.—The evolution of microorganism defense systems has led to intensive searches for new drugs extracted from various natural products to fight microbial infections. This study evaluated the antibacterial and antifungal activity of *Lygodium venustum*, a climbing fern. A phytochemical screening was performed using ethanol extract from leaves of *L. venustum* (EELV), detecting the presence of phenols, tannins, flavonoids and alkaloids. The test of Minimal Inhibitory Concentration (MIC) against *Escherichia coli*, *Staphyloccocus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. krusei* and *C. tropicalis* was evaluated using the microdilution method, resulting in inhibitory concentrations ≥ 1024 µg/mL. Using a subinhibitory concentration of 128 µg/mL of EELV, the modulatory potential of the extract was tested against multidrug-resistant clinical isolates, resulting in synergism when combined with Gentamicin and actually altering the phenotype of *S. aureus* from sensitive to resistant. The extract also increased the effect of the kanamycin against *S. aureus*. This was the first report of modulatory antibiotic activity by a member of *Lygodium*.

Key Words.—*Lygodium venustum*, microdilution, antimicrobial, modulator

Microbial infectious diseases have prompted the development of studies to understand their drug resistance mechanisms and the creation of drugs to avoid these defenses. Infection by *Staphylococcus aureus* is among the most common problems in hospitals due to its resistance against several antibiotics. *Pseudomonas aeruginosa* is the cause of nosocomial infections, particularly in people with cystic fibrosis. *Escherichia coli* is commonly found in the intestinal tract, but certain strains have been closely linked to serious urinary tract infections and diarrhea (Tortora *et al.*, 2008). *Klebsiella pneumonia*, although confined to the normal flora, has emerged as an important hospital pathogen capable of causing severe morbidity and mortality in pediatric patients (Pfaller *et al.*, 1998). Strains of *Candida* have concerned the medical community due to their role in high-morbidity and mortality infections,

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particularly in immunocompromised patients (Richardson and Lass-Florl, 2008; Coutinho, 2009).

Through natural selection plants have developed several mechanisms against parasitism and herbivory. The production of defensive chemical compounds, such as secondary metabolites, indicates evolutionary adaptive responses from the pressure caused by these ecological relationships (Rhodes, 1994). Products derived from plants that feature antimicrobial properties or the ability to improve the antimicrobial potential of existing drugs play an important role in battling infectious diseases (Coutinho *et al.*, 2009). They can serve as alternative therapeutic agents with the ability to directly counter natural microbial resistance to drugs.

Lygodium venustum is a fern with a pantropical distribution used as a bioindicator of degraded environments (Mehltreter, 2006). This fern is used as a medicinal plant in Latin America due to its antifungal, trichomonacidal, antidiarrheal, anti-inflammatory and analgesic activity (Duke and Ottesen, 2009; Argueta *et al.*, 1994). It is used in the Peruvian Amazon as an adaptogen and as an ingredient of the sacred beverage "ayahuasca" (Rivier and Lidgren, 1972). Few studies have reported on the bioactivity of *L. venustum* in preclinical studies (Alanis *et al.*, 2005; Calzada *et al.*, 2007; Calzada *et al.*, 2010), as is true in others ferns (Xavier, 2007).

In this work, a phytochemical screening was performed on the ethanol extract from leaves of *L. venustum*; its antimicrobial activity was assayed against bacterial and fungal strains, as well the modulatory potential against aminoglycosides and antifungal drugs.

Material and Methods

Plant Material

Leaves of *L. venustum* were collected in the city of Crato, Ceará, Brazil. The plant was identified by Dr. Antonio Álamo Feitosa Saraiva and voucher specimens were deposited at the Herbarium Caririense Dárdano de Andrade-Lima of the Regional University of Cariri – URCA, under number 5569 HCDAL.

Preparation of Ethanol Extract from Leaves of *L. venustum* (EELV)

The leaves were partially milled and 211.18 g of powdered material was extracted by maceration using 1 L of 95% ethanol as solvent at room temperature. The mixture was allowed to stand for 72 h at room temperature. The extract was then filtered and concentrated under vacuum in a rotary evaporator at 60°C and 760 mm/Hg, yielding 103.9 g (Brasileiro *et al.*, 2006).

Phytochemical characteristics

The phytochemical assays were used for the qualitative analysis of the presence of secondary metabolites. The detection tests to evaluate the presence

TABLE 1. Bacterial source and antibiotic resistance profile.

Bacteria	Source	Antibiotic resistance
Escherichia coli 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
Staphhylococcus aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
Pseudomonas Aeruginosa 03	Urine culture	Cpm, Ctz, Imi, Cip, Ptz, Lev, Mer, Ami

Ast-Aztreonam; Ax-Amoxacillin; Amp-Ampicillin; Ami-Amicilina; Amox-Amoxillin, Ca-Cefadroxil; Cfc-cefaclor; Cf-Cephalothin; Caz-Ceftazinidima; Cip-Ciprofloxacin; Clo-ChlorampKenicol; Imi-Imipenem; Can-Canamycin; Szt-Sulfametrim; Tet-Tetracycline; Tob-Tobramycin; Oxa-Oxacillin; Gen-Gentamicin; Neo- Neomycin; Para- Paramomicina; But-Butirosina; Sis-Sisomicin; Net-Netilmicin; Com-Cefepime; Ctz-Ceftazidime; Ptz-Piperacilina-tazobactam; Lev-Levofloxacina; Mer-Merpenem.

of heterosides, saponins, phenols, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids were performed according to the method described by Matos (2009). The tests are based on the visual observation of color modifications and formation of precipitate after the addition of specific reagents.

Microbial strains

The bacteria used in the Minimal Inhibitory Concentration (MIC) test were the standard strains of *E. coli* ATCC25923, *S. aureus* ATCC10536, *P. aeruginosa* ATCC15442 and *K. pneumonia* ATCC4362. The antifungal assays used standard strains of *Candida albicans* ATCC40006, *C. krusei* ATCC2538 and *C. tropicalis* ATCC40042. To evaluate the modulatory activity of the extract, the following multi-resistant bacterial strains were used, isolated from clinical environments: *P. aeruginosa* 03, *E. coli* 27 and *S. aureus* 358, with the resistance profile demonstrated in Table 1 and the same fungal strains used in the MIC test. All strains were obtained from the Laboratory of Clinical Mycology – UFPB.

Drugs

The drugs used in the tests were the aminoglycosides kanamycin, amikacin, neomycin and gentamicin, and antifungals mebendazole, amphotericin B, nystatin and benzoyl metronidazole (Sigma Co., St. Louis, USA). All drugs were diluted in sterile water.

Minimal Inhibitory Concentration

Broth microdilution was the method used. The EELV solution was dissolved using DMSO and diluted to 1024 μg/mL using sterile distilled water. The bacterial inoculum was diluted using BHI to a final concentration of 10^5 CFU/mL. A total of 100 μL of each inoculum was distributed in each well of a microtiter plate with 96 wells, and then submitted to a twofold serial dilution

TABLE 2. Phytochemical characterization of ethanol extracts of *L. venustum*.

Metabolites														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
+	−	+	−	−	+	+	+	+	−	+	−	−	+	+

1 – Phenols; 2 – Tannin pyrogallates; 3 – Tannin Phlobaphenes; 4 – Anthocyanidins; 5 – Anthocyanins; 6 – Flavones; 7 – flavonols; 8 – Xanthones; 9 – Chalcones; 10 – Aurones; 11 – Flavononls; 12 – Leucoanthocyanidins; 13 – Catechins; 14 – Flavonones; 15 – Alkaloids; (+) presence; (−) absence.

using 100 µL of the extract, with concentrations ranging between 8 and 512 µg/mL. The plates were incubated for 24 hours at 35 °C (Javadpour *et al.*, 1996). Bacterial MIC was determined using resazurin, while the MIC of fungi was determined by turbidity. The MIC was defined as the lowest concentration where no growth can be observed, according to NCCLS (2008).

Drug Modulation Test

To observe whether the extract would alter the action of antimicrobial drugs against the tested strain, the method proposed by Coutinho *et al.* (2008) was used. The EELV was tested at a sub-inhibitory concentration (MIC/8 = 128 µg/mL). A 100 µL sample of a solution containing BHI, the microbial inoculums and extract were placed in each well. After this, 100 µL of the antimicrobial drug was mixed with the first well, following the twofold dilution. Concentrations of aminoglycosides and antifungals ranged between 2.44 and 2500 µg/mL and 2 to 512 µg/mL, respectively.

RESULTS

The phytochemical characterization showed the presence of phenols, tannins, flavonoids and alkaloids, as shown in Table 2.

The antibacterial and antifungal assays of EELV did not demonstrate clinically relevant results, with MICs ≥1024 µg/mL. However, when the modifying activity of EELV against aminoglycosides was evaluated, the Gram-negative *E. coli* 27 and Gram-positive *S. aureus* 358 strains showed synergistic activity when combined with gentamicin and kanamycin (Table 3). The combination of the extract with antifungals did not show any modulatory activity against strains of *Candida*.

DISCUSSION

Several plants used in the religious beverage "ayahuasca", such as *L. venustum*, contain alkaloids (Rivier and Lidgren, 1972). This fact is corroborated by the results of our phytochemical screening. Other species from the genus *Lygodium* have been the subjects of more detailed chemical studies, including the isolation of some compounds (Zhang *et al.*, 2005;

TABLE 3. Antibacterial activity modulating the ethanol extract from leaves of *L. venustum* (μg/mL).

	Modulation of antibiotic for EELV			
	E. coli 27		*S. aureus* 358	
Extract/ Antibiotic	MIC combined	MIC alone	MIC combined	MIC alone
EELV/ Kanamycin	156.25	156.25	39.06	156.25
EELV/ Amikacin	312.5	312.5	78.125	78.125
EELV/ Neomycin	156.25	156.25	39.06	39.06
EELV/ Gentamicin	39.06	1250	2.44	19.53

MIC: Minimal Inhibitory Concentration; EELV: Ethanol Extract of *L. venustum*.

Kurumatani *et al.*, 2001; Achari *et al.*, 1986). However, this is the first work to focus on the chemical composition of *L. venustum*.

The lack of the antibiotic activity of *L. venustum* against strains of *E. coli* was verified in another report (Alanis *et al.*, 2005). The results demonstrate that the extracts were not efficient inhibitors of bacterial growth, as their inhibition percentages were lower than 50%. A relevant note regarding this research is the value of the extract concentration used in the test, 8 mg, which is considered high for clinical applications (Houghton *et al.*, 2007), as demonstrated in our work. Additionally, it is important to note that the microdilution method used in present study is currently the preferred technique to evaluate antimicrobial activity, compared to other methodologies using agar diffusion (Greger and Hadacek, 2000).

The methanol extracts of other plants from the genus *Lygodium* such as *Lygodium japonicum* (Thumb.) SW. was tested against strains of *P. aeruginosa*, *S. aureus, E. coli* and *C. albicans* using the disk diffusion method, impregnated with 40 μg of dried plant material/disk, but no bioactivity was demonstrated (Taylor *et al.*, 1995). Our results corroborate those obtained in this work by Taylor *et al.* (1995).

Compared with the isolated action of drugs, EELV increased the antibiotic activity of amikacin against *S. aureus.* When associated with gentamicin, it demonstrated a very promising modulatory activity against *E. coli* and *S. aureus*, causing a reversal of the resistant phenotype of this strain to sensitive according to the classification of NCCLS (2005). The observed bioactivity of the extract in combination with the antibiotics may indicate that secondary metabolites such as tannins, flavonoids and alkaloids –all secondary metabolites with well-known antimicrobial activity and found in the EELV– could be acting in association with the assayed drugs, enhancing the activity of these drugs at lower concentrations (Scalbert 1991; Bylka *et al.*, 2004; Zongo *et al.*, 2009).

This is the first report on the modulatory activity against aminoglycosides by a fern. This activity indicates the possibility of development of new drugs derived from the association between natural products isolated from *L. venustum* with antibiotics, to be used in antibiotic therapy against multi-drug resistant bacteria, as well as prevent the emergence of drug resistant bacteria.

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